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Cell Biology

Efficient gene transfer into human hepatocytes by baculovirus vectors

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ABSTRACT **Viral vectors are the most efficient tools for gene delivery, and the search for tissue-specific infecting viruses is important for the development of *in vivo* gene therapy strategies. The baculovirus *Autographa californica* nuclear polyhedrosis virus is widely used as a vector for expression of foreign genes in insect cells, and its host specificity is supposed to be restricted to arthropods. Here we demonstrate that recombinant *A. californica* nuclear polyhedrosis virus is efficiently taken up by human hepatocytes via an endosomal pathway. High-level reporter gene expression from heterologous promoters was observed in human and rabbit hepatocytes *in vitro*. Mouse hepatocytes and some other epithelial cell types are targeted at a considerably lower rate. The efficiency of gene transfer by baculovirus considerably exceeds that obtained by calcium phosphate or lipid transfection. These properties of baculovirus suggest a use for it as a vector for liver-directed gene transfer but highlight a potential risk in handling certain recombinant baculoviruses.**

Gene therapy is a powerful concept for the treatment of a variety of diseases (1–3). The liver is the organ where many genetic diseases are manifested (4). The first clinical trial for the treatment of familial hypercholesterolemia was based on transfer of the low density lipoprotein receptor gene to hepatocytes *in vitro* and subsequent retransplantation (5). Since this protocol is very complicated and probably does not result in sufficient therapeutic effects, it would be particularly important to have methods available that allow for gene transfer to the liver *in vivo* (6, 7). Retroviruses integrate their genome into that of the host cell but only in dividing cells (8); therefore, gene transfer to the liver requires stimulation of cell proliferation—e.g., by partial hepatectomy (9–11). Adenoviral vectors deliver genes to the liver at very high efficiencies, approaching 100% gene transduction to hepatocytes (12). However, the major disadvantage of this type of vector is the instability of the transferred genes in the target cells due to a lack of integration and to immunological response (13). Recent modifications of the adenoviral viral vector system leading to a reduction of the immune response resulted in a significant prolongation of function of the transferred gene (14). The general disadvantage of viral vectors available so far for gene delivery to the liver is their lack of organ specificity.

Alternative means for gene targeting to the liver have been developed, which are based on the concept of receptor targeting (15–21). Despite the fact that the generation of particles for receptor targeting has been optimized recently and a remarkable efficiency of *in vivo* gene transfer could be achieved (22), this is still not efficient enough for therapeutic application. Thus, it would be important to investigate the abilities of other viruses with regard to hepatocyte-specific gene delivery.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV), which is used as a vector for protein overproduction in insect cells (23–25), was also studied in the past regarding its ability to infect mammalian cells (26–29). Abortive infection of some cell types by AcNPV has been detected, but neither gene expression nor viral DNA replication could be observed, most likely due to the restriction of viral promoter function to insect cells. These results suggested that the virus would not be a risk factor for humans and even prompted the development of baculovirus vectors as a biological weapon against particular insects. Recently, a baculovirus expressing an insect-specific toxin has been used in a field trial (30).

As infection of liver cells by AcNPV was not tested before, we designed recombinant baculoviruses that should express reporter genes provided the virus is taken up by hepatocytes. The results described in this report favor the use of baculovirus vectors for the development of strategies for liver-directed gene therapy but also point at a requirement for a careful investigation of the potential risks connected with the unrestricted use of this type of virus.

MATERIALS AND METHODS

Generation of Recombinant Baculoviruses. Recombinant baculoviruses were constructed using the transfer vector pVL1392 (31) (PharMingen). For AcNPV-PHTag the gene coding for a C-terminally truncated simian virus 40 (SV40) large tumor antigen (T antigen) (32) was cloned next to the viral polyhedrin (PH) promoter, whereas for AcNPV-CMVTag this gene was fused to the cytomegalovirus (CMV) immediate early promoter (33) and inserted into pVL1392 in the orientation opposite to the PH promoter. The *Photinus pyralis* luciferase reporter gene (34) was introduced into the baculovirus vector either under control of the PH promoter (AcNPV-PHL) or under control of the CMV immediate early promoter (AcNPV-CMVL) fused to the luciferase gene before cloning into the transfer vector. Viruses were generated according to standard procedures (23, 35). Budded virus was concentrated from cell culture medium by sedimentation at 35,000 × *g* for 30 min and purified by centrifugation in a 24–62% (wt/vol) linear sucrose gradient. The virus titer was estimated by plaque assay on Sf9 cells.

Cell Culture. Primary human hepatocytes were obtained by perfusion with collagenase of small sections of normal tissue from resected livers of patients with liver metastases of colon carcinoma as described (36). Primary mouse or rabbit hepatocytes were obtained by perfusion with collagenase of livers

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; SV40, simian virus 40; T antigen, tumor antigen; CMV, cytomegalovirus; moi, multiplicity of infection; PH, polyhedrin.

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in situ. Primary hepatocytes were seeded onto a layer of collagen IV and grown in William's E medium without serum. Most experiments were carried out using an established hepatocyte line (Huh7) derived from a human hepatocellular carcinoma (37), which was grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum. A number of cell lines from different tissue origin were used (given in the legend to Fig. 3) and grown in the media recommended by the authors or by the American Type Culture Collection. Treatment with baculovirus was done 48 h after seeding.

Incubation of Mammalian Cells with Recombinant Baculovirus. Different cell types were treated with baculovirus under similar conditions. Incubation of established cells was generally done in complete medium containing 10% (vol/vol) heat-inactivated fetal calf serum for 1 h at 37°C at the multiplicity of infection (moi) indicated. Untreated serum caused a considerable decrease in gene transfer efficiencies. Primary hepatocytes were treated with virus in the absence of serum. Treatment with a maximal virus concentration of 1500 moi per cell did not cause any toxic side effect as indicated by trypan blue exclusion and an unchanged plating efficiency at 2 days after treatment.

Assays for Reporter Enzyme Activities. Cells were lysed in a buffer containing 100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, and 1 mM dithiothreitol. For luciferase assays, 50 μl of cleared lysate was incubated with 180 μl of reaction buffer containing 25 mM phosphate buffer (pH 7.8), 4 mM EGTA, 15 mM $MgSO_4$, 1 mM dithiothreitol, and 1 mM ATP, and 50 μl of a 20 μM luciferin solution was injected. Relative light units were measured using a luminometer (Berthold, Wildbad, Germany). Values given in the figures are those obtained from extracts of 10^4 cells. A background value of 120 relative light units was substracted in all cases. Assays for β-galactosidase activity were carried out using the Galacto-Light kit (Tropix, Bedford, MA) essentially as described by the manufacturer.

RESULTS

Baculovirus Infection of Hepatocytes. To explore the possibility that a recombinant baculovirus might be used to infect human hepatocytes, we constructed recombinant baculoviruses with the gene coding for a truncated large T antigen of SV40 under the control of either the PH gene promoter of baculovirus or the immediate early promoter of CMV. After incubation of human hepatocellular carcinoma cells (Huh7) for 1 h with the virus at a moi of 1000, T antigen was visualized by immunofluorescence. Whereas nuclei of hepatocytes treated with AcNPV-PHTag were negative, those treated with AcNPV-CMVTag were strongly positive (Fig. 1). The efficiency of obtaining T antigen-positive nuclei by treatment of Huh7 cells with the latter virus was usually 80–100% at a moi 1000 and still ≈50% at a moi of 100. The actual uptake of both types of recombinant baculoviruses was confirmed by electron microscopy (Fig. 2).

Specific Uptake by Hepatocytes. To investigate the specificity of hepatocyte infection by baculovirus, we tested different hepatic and nonhepatic cell types for expression of the luciferase reporter gene. We could not detect luciferase activity in human or mouse hepatocytes or NIH 3T3 fibroblast cells on infection by AcNPV-PHL, whereas high levels of activity were found in Sf9 insect cells, which shows that the PH promoter is inactive in the mammalian cells tested (Fig. 3*a*). However, if expression was driven by the CMV promoter, high levels of luciferase activity were detected in various hepatic cell types (Fig. 3*b*). In contrast, no or very low levels of expression were found in five lymphocytic cell lines, human and rodent carcinoma lines of different tissue origin (lung, breast, kidney, and bladder), mouse fibroblasts, and various neuronal cells (Fig. 3*b*). The established hepatocarcinoma line Huh7 repro-

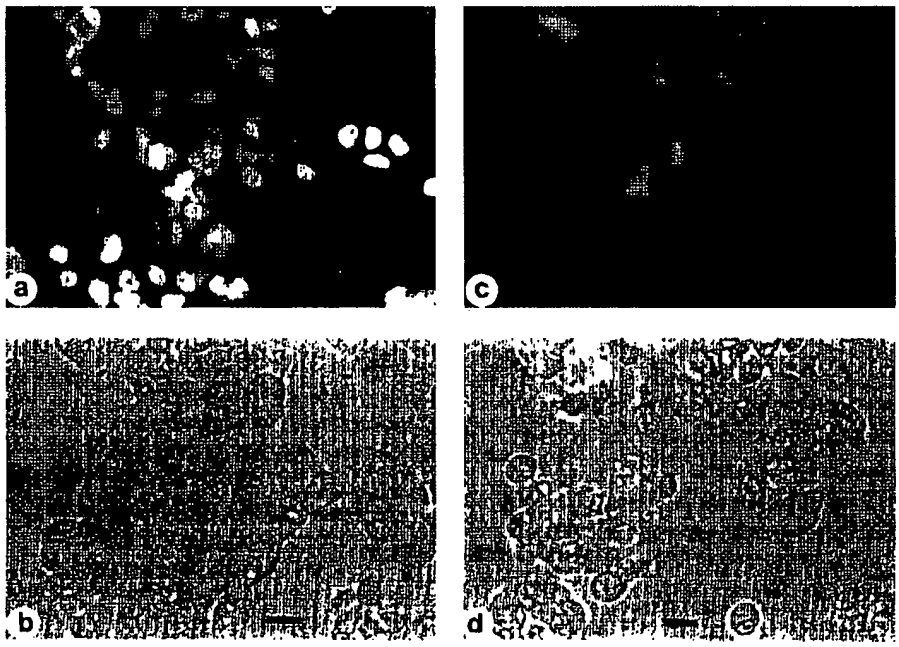

FIG. 1. Immunofluorescence detection of SV40 large T antigen expression in human hepatocytes after infection by recombinant baculoviruses. (*a* and *b*) AcNPV-CMVTag. (*c* and *d*) AcNPV-PHTag. In these recombinants, expression was driven by either the PH promoter or the CMV immediate early promoter. Huh7 human hepatocarcinoma cells (37) grown on coverslips were incubated with virus at a moi of 1000 for 1 h. Thirty-six hours later cells were fixed with methanol/acetone at −20°C for 5 min. For immunofluorescence detection, fixed cells were first incubated for 1 h with the T antigen-specific monoclonal antibody 416, washed, incubated for 1 h with a biotinylated goat anti-mouse antibody, and finally incubated with streptavidin-Texas Red. (*a* and *c*) Immunofluorescence detection. (*b* and *d*) Phase-contrast micrograph. (Bars = 25 μm.)

FIG. 2. Detection of baculovirus particles in human hepatocytes by electron microscopy. Huh7 human hepatocarcinoma cells were infected with the recombinant baculovirus vector AcNPV-CMVTag at a moi of 100 for 1 h. After 2 h cells were washed with PBS, scraped off from the plate, fixed with 2% glutaraldehyde, and embedded in Epon 812. Thin sections were cut on a Ultrotom (LKB). Analyses were carried out, and micrographs were taken using a JEM 100 CX (JEOL) electron microscope. Arrowheads indicate the location of baculovirus particles; cytoplasm (c), nucleus (n), and nuclear membrane (nm) are also indicated. (× 63,000.)

ducibly expressed one order of magnitude higher levels of luciferase than the hepatoblastoma line HepG2 or primary human hepatocytes, which, in turn, expressed almost 10-fold higher levels than primary or established mouse hepatocytes. Expression levels in rabbit primary hepatocytes were normally slightly higher than those in human primary hepatocytes. Human liver sinusoidal endothelial cells also express some luciferase activity (Fig. 3*b*).

Mode of Baculovirus Uptake by Hepatocytes. Since uptake of baculovirus by hepatocytes appeared to be strikingly efficient and specific, we investigated the mechanism of uptake in more detail. Treatment of Huh7 cells with AcNPV-CMVL, tested at different moi, produced a clear dose–response curve (Fig. 4*a*), suggesting a saturable receptor-mediated uptake of the virus. Competition experiments (Fig. 4*b*) were carried out in these cells using a constant amount of the expressing virus AcNPV-CMVL (moi of 50) and increasing amounts of the nonexpressed virus AcNPV-PHL. Both monolayers and freshly trypsinized cells were infected. At a ratio of AcNPV-CMVL to AcNPV-PHL (moi) of 1:15 (total moi of 800), there was no competition for the expressing virus in cells in the monolayer; some competition was, however, observed at higher ratios (1:30). However, in the trypsin-treated cells, competition was effective at lower ratios (above 1:3), suggesting that the number of receptors for the virus had been considerably reduced. Chloroquine had a very strong effect on luciferase expression (Fig. 4*c*), suggesting that endosomal maturation is required for transport of the virus as was shown previously for insect cells (45).

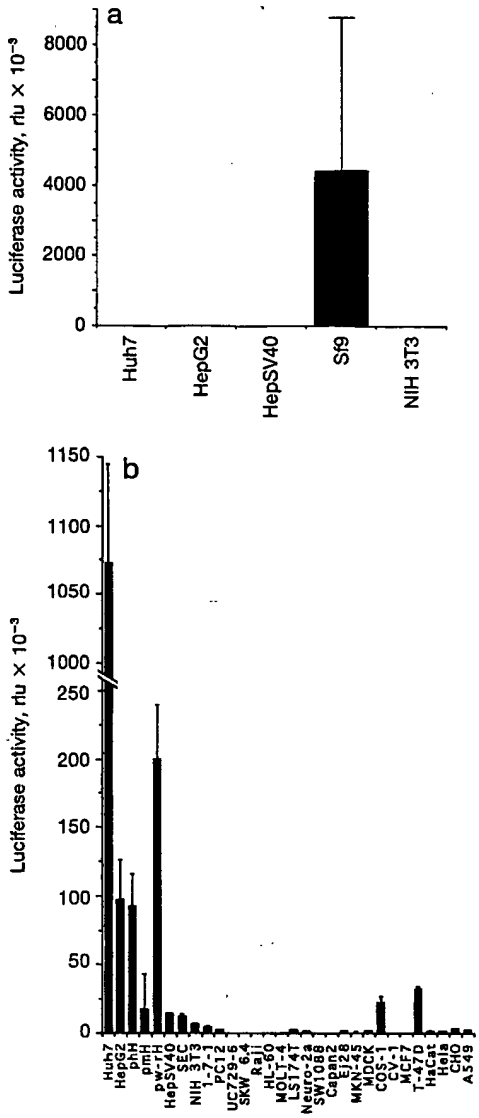

FIG. 3. Cell-type specific infection and luciferase gene expression by recombinant baculoviruses. (*a*) Infection with AcNPV-PHL. Expression of the luciferase gene was driven by the PH promoter. (*b*) Infection with AcNPV-CMVL. Expression was driven by the CMV immediate early promoter. The following cell lines were used: *Spodoptera frugiperda* insect cell line Sf9, human hepatocarcinoma line Huh7 (37), human hepatoma line HepG2 [American Type Culture Collection (ATCC)], a clone (HepSV40) of hepatocytes obtained from an SV40 T antigen-expressing transgenic mouse (38), human liver sinusoidal endothelial cells (SEC) immortalized by polyoma virus large T antigen (39), parental NIH 3T3 cells and a clone (1-7-1) expressing both subunits of the asialoglycoprotein receptor (40), human lymphocyte lines HL60, MOLT-4, UC729-6, and SKW 6.4, Burkitt lymphoma cells Raji, rat adrenal pheochromocytoma cell line PC12, human colon carcinoma line LS174T, mouse neuroblastoma Neuro-2a, human astrocytoma line SW 1088, human pancreatic adenocarcinoma Capan 2, canine kidney epithelial cells MDCK, African green monkey kidney epithelial cells CV-1, human mammary adenocarcinoma cells MCF7, mammary ductual carcinoma cells T47-D, human cervix carcinoma cells HeLa, Chinese hamster ovary (CHO) cells, and human lung carcinoma cells A549 (all from ATCC), human bladder carcinoma cells Ej28 (41), human gastric carcinoma cells MKN-45 (42), SV40-transformed CV-1 cells COS-1 (43), human immortalized keratinocytes HaCat (44), primary human hepatocytes (phH), primary hepatocytes from Watanabe rabbit (pw-rH), and primary mouse hepatocytes (pmH). Luciferase expression was analyzed 36 h after infection. Values of relative light units (rlu) given refer to extracts from 10^4 cells.

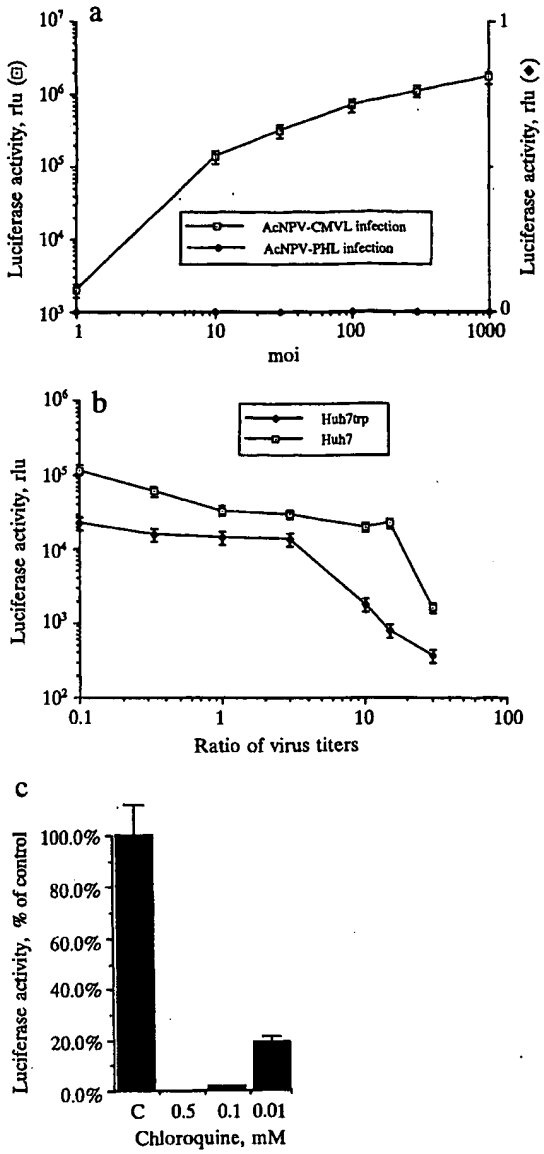

FIG. 4. Effect of virus titer, virus competition, and inhibition of endosomal maturation on baculovirus-mediated expression of the luciferase gene in Huh7 cells. (*a*) Effect of virus titer on luciferase activity. (*b*) Influence of competing nonspecific AcNPV-PHL virus on expression levels obtained with AcNPV-CMVL. (*c*) Effect of chloroquine treatment of cells. (*a*) Huh7 cells were infected with AcNPV-CMVL or AcNPV-PHL at increasing moi. (*b*) Huh7 cells were infected with AcNPV-CMVL at a fixed moi of 50 and increasing amounts of AcNPV-PHL. (*c*) Huh7 cells were infected with AcNPV-CMVL at a moi of 100. In the case of trypsin treatment, cells were incubated with trypsin (500 μg/ml) for 3 min at 37°C, washed, and replated 1 h before virus application (Huh7trp cells). For treatment with chloroquine, cells were incubated with the agent for 4 h (3 h before infection and during the infection time of 1 h). Control cells (C) were incubated with chloroquine 12 h after infection. Expression was analyzed 36 h after infection as described in *Materials and Methods*. rlu, Relative light units.

Efficiency of Gene Transfer into Hepatocytes. To gain some insight into the efficiency of gene transfer by a baculovirus-derived vector, a comparative study was carried out in Huh7 cells including calcium phosphate transfection, lipid transfection (lipofection), and adenoviral vector infection. The β-galactosidase reporter gene under control of the Rous sarcoma virus promoter was used to allow for quantification of both the total activity in the cells treated and the percentage of expressing cells. The results are summarized in Table 1. The data clearly show that both viral vector systems are more efficient than calcium phosphate transfection or lipofection if used at a moi of 100. At the time of the assay (36 h after treatment), which is optimal for evaluating transient expression, total reporter enzyme activity was higher with the baculovirus vector, whereas the actual number of expressing cells was higher with the adenoviral vector. Whereas adenoviral vectors are toxic at moi >100, baculovirus can be used at a moi of 1000 without problems, resulting in almost 100% gene transduction efficiency (see Fig. 1). Baculovirus-transduced genes remain highly active over a period of at least 2 weeks in nondividing primary hepatocytes but decline by more than one order of magnitude in growing established hepatocytes (V.S. and C.H., unpublished observations).

Table 1. Comparison of various gene delivery methods regarding transfer and expression efficiencies in human hepatocytes

Method	moi	β-Galactosidase activity, rlu per 10^3 cells	Positive cells, %
Calcium phosphate		81,200 ± 7,400	20
Lipofectamine		72,300 ± 6,800	15
Baculovirus	10	94,600 ± 8,400	12
	100	512,300 ± 62,600	50
Adenovirus	10	53,100 ± 4,600	30
	100	151,800 ± 17,200	95

Three-centimeter dishes were seeded with 1×10^5 Huh7 cells the day before treatment. Four dishes were used for every method. Calcium phosphate transfection was according to standard protocols (41) and lipofection using either Lipofectin or Lipofectamine (GIBCO/BRL/Life Technologies) was done according to the protocol provided by the manufacturer. Infection with either baculovirus or adenovirus vectors was done for 1 h at the moi given. The adenovirus harboring a Rous sarcoma virus promoter-driven gene coding for nuclear β-galactosidase was a kind gift of Andre Lieber and Mark Kay (University of Washington, Seattle). β-Galactosidase assays were done as described in *Materials and Methods*. Extract from every dish was tested for activity, and the mean values of three dishes are given. Histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside was carried out in parallel dishes. The number of blue cells was counted under the microscope and is given as a percentage of the total number of cells. Three separate areas of a dish containing 1000 cells were counted. rlu, Relative light units.

DISCUSSION

Our results demonstrate that a recombinant baculovirus can efficiently infect human hepatocytes and can deliver functional genes to the nucleus. Using a promoter that is known to be active in mammalian cells, we were able to show that human hepatocytes infected by a recombinant virus express foreign genes at high levels. These levels obtained with baculoviruses containing test genes under control of the CMV promoter are much higher than those obtained by calcium phosphate coprecipitation or lipofection. The high efficiency and the degree of specificity of gene transfer into hepatocytes due to the presence of a particular receptor on hepatocyte cell membranes and inhibition of gene expression by inhibition of endosomal maturation suggest involvement of the endocytotic pathway in the uptake route used by baculovirus in hepatocytes. It was postulated at the beginning of this work that a candidate receptor could be the asialoglycoprotein receptor, since glycosylated baculovirus envelope proteins are missing terminal sialic acid residues and might, therefore, be recognized as degraded glycoproteins. However, a derivative of NIH 3T3 cells (1-7-1) expressing the cloned asialoglycoprotein

receptor (40) did not show significant uptake of luciferase-expressing virus (see Fig. 3*b*), and asialoorosomucoid did not compete with the virus for uptake by hepatocytes (data not shown). Thus, if there is a receptor for binding of baculovirus to, and uptake by, hepatocytes, it remains to be identified. From our experiments, it is obvious that human and mouse hepatocytes differ considerably regarding the efficiency of baculovirus uptake, which may well be due to the structure or abundance of the potential receptor.

Our findings may lead to the development of a new type of vector for liver-directed gene therapy. If compared with the existing tools for gene transfer to hepatocytes, recombinant baculovirus is comparable to both artificial receptor-targeting particles and retroviral vectors regarding the efficiency of gene transfer *in vitro* (unpublished results) and is even comparable to adenovirus vectors with regard to expression levels. However, baculovirus is more specific for hepatocytes than adenovirus and has the enormous advantage of most likely not expressing its own viral genes in mammalian cells due to the restricted function of its promoters. Like adenovirus, baculovirus normally does not integrate into the host genome (unpublished data); hence, it can only be considered as a short-term expression vector so far. However, since stable expression was observed in resting primary hepatocytes over >2 weeks, longer lasting effects might be possible in liver tissue *in vivo*.

Furthermore, our results emphasize possible hazards for humans in the unrestricted use of baculovirus as vectors for gene expression in insect cells *in vitro* or in insects *in vivo*. Our data have clearly shown that in hepatocytes there is no detectable foreign gene expression from the PH promoter, which, in most cases, is used for foreign gene expression in insect cells and is tightly regulated for late gene expression. Thus, properly designed vectors for insect cells should not harm investigators, even if their livers should somehow become infected. However, often little care is taken in constructing recombinants, and (for example, during cloning) prokaryotic DNA sequences remain in the final construct because they are thought to pose no disadvantage in insect cells. Those or even some baculoviral sequences may function as promoter elements under certain circumstances as was shown for the T7 promoter (46). For both risk assessment and application to gene therapy, further investigations on the efficiency of liver targeting and gene expression *in vivo* are required. If studies in various animal models *in vivo* could confirm the striking ability of baculovirus to selectively infect hepatocytes and immunological problems could be ruled out, this would represent important progress in the development of liver-directed gene therapy.

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